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In Vivo Competence of Murine Cytomegalovirus under the Control of the Human Cytomegalovirus Major Immediate-Early Enhancer in the Establishment of Latency and Reactivation^{∇†}

Montse Gustems,¹ Andreas Busche,² Martin Messerle,² Peter Ghazal,³ and Ana Angulo^{1*}

Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona 08036, Spain¹; Department of Virology, Hannover Medical School, Hannover 30625, Germany²; and Division of Pathway Medicine, University of Edinburgh, 49 Little France Crescent, Edinburgh EH16 4SB, United Kingdom³

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The human cytomegalovirus (HCMV) major immediate-early enhancer has been postulated to play a pivotal role in the control of latency and reactivation. However, the absence of an animal model has obstructed a direct test of this hypothesis. Here we report on the establishment of an in vivo, experimentally tractable system for quantitatively investigating physiological functions of the HCMV enhancer. Using a neonate BALB/c mouse model, we show that a chimeric murine CMV under the control of the HCMV enhancer is competent in vivo, replicating in key organs of mice with acute CMV infection and exhibiting latency/reactivation features comparable for the most part to those of the parental and revertant viruses.

Reactivation of human cytomegalovirus (HCMV) in an immunocompromised host frequently leads to a variety of severe complications, such as pneumonia, hepatitis, and retinitis (30). The HCMV major immediate-early promoter (MIEP) has been regarded as a key genetic element in determining the commencement of lytic infection and the switch from latency to reactivation (17, 35, 36). The MIEP steers the extent and patterns of expression of the MIE genes, which encode multifunctional proteins required for the productive replication cycle (25, 28). The enhancer region of the MIEP is controlled by a complex interplay between host factors and virally encoded proteins (12, 36). Thus, binding sites for multiple signal-regulated cellular factors, such as NF- κ B, CREB/ATF, Sp1, AP-1, YY1, Ets, RAR/RXR, and serum response factors, lie in this regulatory region. The importance of the HCMV MIEP enhancer in the context of the infection of cultured cells has been documented (15, 18, 20, 26, 27). However, the lack of an animal model system that sustains significant HCMV replication has prevented the assessment of the role and mechanisms of action of this region during latent infection. Thus, there is an urgent need to develop in vivo models to address this issue.

Infection of mice with murine CMV (MCMV) has proven to be an invaluable model for studying aspects of the biology of CMV infection. The MCMV MIE locus resembles in many ways its HCMV counterpart, and significant information has been drawn from this system concerning MIE gene functions and MIEP regulation (8, 33). In this context, we have described the absolute requirement of the MCMV enhancer for productive infection in its natural host (13). While the primary sequence and architecture of the MCMV and HCMV enhancers are quite different, they share many of the same signal-regu-

latory control elements (7, 10, 12), conferring both similar and distinct biological properties to them. Accordingly, the first attempts to study HCMV MIEP function in an intact physiological system involved developing murine transgenic models using an HCMV enhancer linked to a reporter gene (3, 4, 23). However, while informative, these models place the enhancer out of its natural environment of the viral genome and most importantly out of the context of an infection. For these reasons, we sought to address HCMV-enhancer-related questions during viral infection in an in vivo setting by generating the first chimeric humanized MCMV (hMCMV) in which the HCMV enhancer precisely replaces the MCMV enhancer (2). We showed that this enhancer swap virus replicated in permissive NIH 3T3 cells with wild-type kinetics. These observations were extended by Grzimek et al. (14), who used an independent hybrid virus (mCMVhMIEPE) in which the complete MCMV promoter was replaced by both the enhancer and the core promoter of HCMV; this recombinant virus showed normal growth in liver but a partial defect in dissemination or replication within other tissues in immunodepleted BALB/c mice. However, these two enhancer swap viruses were generated on the basis of MCMV genomes lacking one or more viral immunomodulatory genes, which thus led to an attenuated phenotype of the resulting viruses in vivo (24, 37). This deficiency made the quantitative study of the acute infection difficult and greatly impeded the possibility of investigating latency and reactivation issues.

In this report, we have sought to establish a robust in vivo model for studying HCMV enhancer functions in the context of an acute and latent infection. For this purpose, we used a new chimeric virus (hMCMV-ES, where ES signifies enhancer swap [5]) engineered from a full-length MCMV-bacterial artificial chromosome (BAC) genome (37) through recombination techniques in *Escherichia coli* (6). In this recombinant virus, sequences from nucleotides –48 to –1191 of the native MCMV enhancer were replaced by the paralogous sequence from nucleotides –52 to –667 of the HCMV enhancer (Fig.

* Corresponding author. Mailing address: Institut d'Investigacions Biomèdiques August Pi i Sunyer, C/Villarroel 170, Barcelona 08036, Spain. Phone: 34 647 450269. Fax: 34 93 4021907. E-mail: aangulo@ub.edu.

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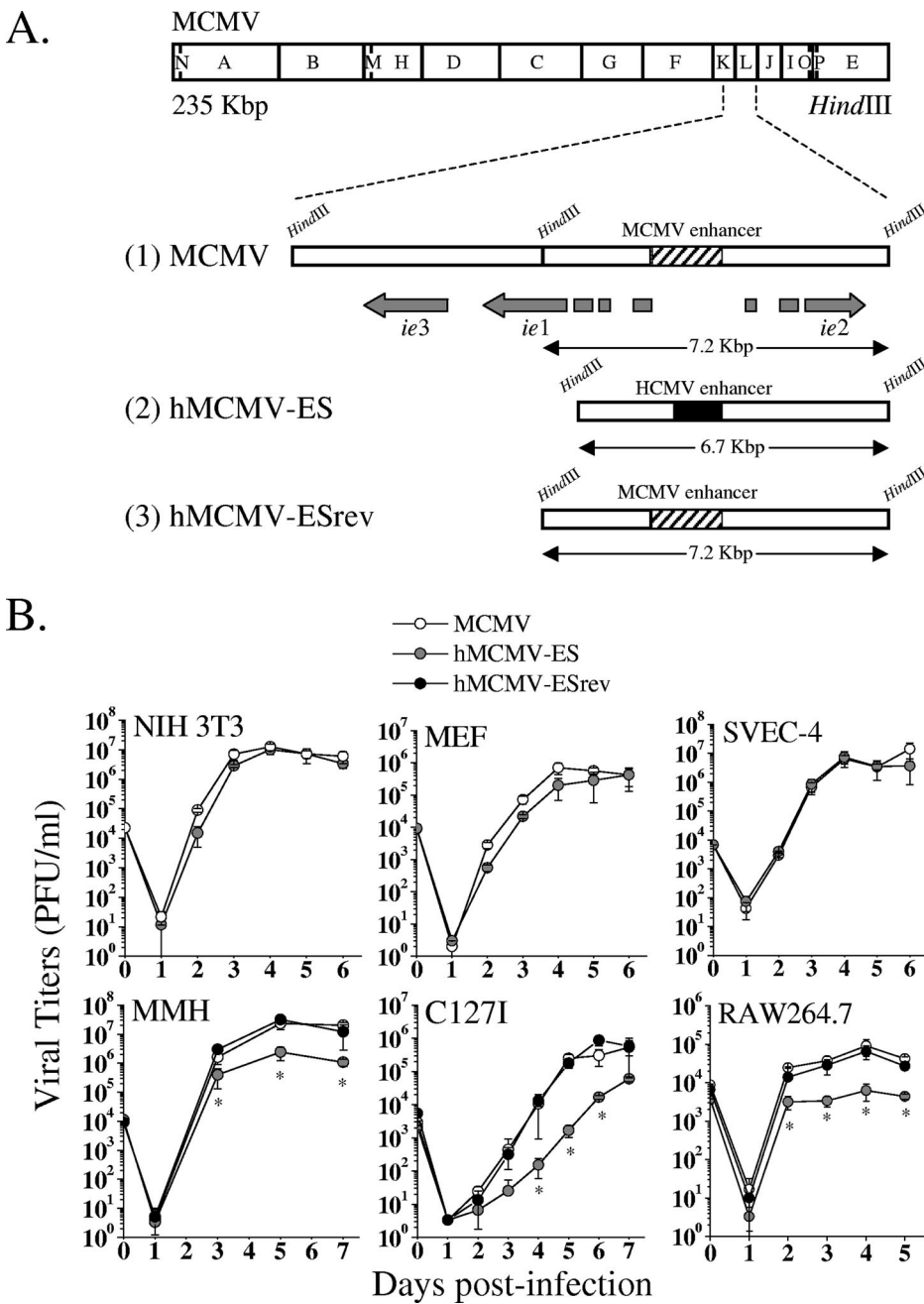


FIG. 1. Genetic organization and in vitro growth analysis of parental MCMV, hMCMV-ES, and hMCMV-ESrev. (A) Schematic illustrations of the parental MCMV, hMCMV-ES, and hMCMV-ESrev genomes. The top line represents the HindIII map of the parental MCMV genome (11), with the HindIII K and L regions enlarged below to show the MIE locus and with the structure of the MIE transcripts (*ie1*, *ie2*, and *ie3*) indicated. Exons of the *ie1-ie3* transcription unit are depicted as gray rectangles. The hatched box represents the MCMV enhancer. Line 1 represents the HindIII fragments K and L from parental MCMV (obtained from the pSM3fr BAC [37]). The hMCMV-ES genome (line 2) was generated by homologous recombination in *E. coli* (6), with the MCMV pSM3fr BAC taken as the parental genome and with MCMV enhancer sequences from nucleotides -48 to -1191 (relative to the *ie1/ie3* MCMV transcription start site) replaced by sequences from nucleotides -52 to -667 (relative to the *ie1/ie2* HCMV transcription start site) of the HCMV enhancer (represented by a black box). In the hMCMV-ESrev genome (line 3), the native sequences of the MCMV enhancer were reintroduced in hMCMV-ES by replacement of the HCMV enhancer. Sizes of the natural and new HindIII L DNA fragments for each recombinant genome are indicated. The illustration is not drawn to scale. (B) Growth kinetics of hMCMV-ES and control viruses in different cell types. NIH 3T3 cells, MEF, SVEC-4 cells, MMH cells (treated for 10 days before infection with 2% dimethyl sulfoxide), and C127I cells were infected at an MOI of 0.025 PFU/cell, and RAW264.7 cells were infected at an MOI of 0.1 PFU/cell, with MCMV, hMCMV-ES, or hMCMV-ESrev. At the designated days p.i., cell supernatants were collected and titrated by standard plaque assays on MEF. Error bars represent standard deviations of the means of results from triplicate cultures. Asterisks denote statistically significant differences between hMCMV-ES-, MCMV-, or hMCMV-ESrev-infected samples (P value < 0.05) determined by Student's *t* test (two-tailed).

1A, lines 1 and 2). As a control, we generated a revertant genome by restoring the MCMV enhancer in hMCMV-ES (hMCMV-ESrev) (Fig. 1A, line 3), transfected hMCMV-ESrev into NIH 3T3 cells, recovered the corresponding virus, and subjected it to three rounds of plaque purification, and a viral stock was produced. The genomic integrity of the three viruses used in the study was verified by HindIII digestion of the viral DNAs (see Fig. S1 in the supplemental material). Further characterization of the recombinant MCMVs was performed by DNA digestion with additional restriction enzymes, PCR analysis, and sequencing across the MIE region manipulated (data not shown). Finally, viral stocks were tested by PCR for the appropriate excision of the BAC vector sequences (see Fig. S2 in the supplemental material), as any remaining BAC sequences in the viral genome could interfere with the natural course of the infection in mice.

We first sought to analyze the growth phenotype of hMCMV-ES in different murine cell types in culture. For this purpose, we infected NIH 3T3 cells, mouse embryo fibroblasts (MEF), an endothelial cell line (SVEC-4), a liver-derived cell line (MMH [1]), an epithelial cell line (C127I), and the macrophagic cell line RAW264.7 with parental MCMV, hMCMV-ES, and hMCMV-ESrev at a low multiplicity of infection (MOI). Figure 1B shows that while no significant growth defects were detected with hMCMV-ES in the first three cell types, a slightly diminished ability (around 1 order of magnitude) to replicate in MMH, C127I, and RAW264.7 cells was exhibited by this recombinant virus. As expected, the revertant virus was identical to the parental virus in growth kinetics in these cell systems. Thus, although the HCMV enhancer was able to complement the native MCMV enhancer in a variety of cultured cells, these data highlight the distinct cell-specific regulation of viral growth by this region.

We next proceeded to analyze whether differential sensitivity also exists in the *in vivo* phenotype of hMCMV-ES in the immunocompetent-BALB/c-mouse model. Groups of 3-week-old mice were infected intraperitoneally (i.p.) with 1×10^6 PFU of hMCMV-ES or the parental or revertant viruses. At 4, 7, and 14 days postinfection (p.i.), viral titers were examined in relevant sites of MCMV replication. As shown in Fig. 2, hMCMV-ES was able to infect and propagate in the spleen, liver, kidneys, lungs, and salivary glands, although it was considerably attenuated (1 to 2 orders of magnitude, depending on the organ) in comparison to the parental and revertant viruses. Similar results were obtained in immunodeficient SCID CB17 mice (data not shown). These data are, overall, consistent with previous observations made with the slightly different hybrid virus mCMVhMIEPE in immunodepleted adult BALB/c mice (14). Altogether, the difference in sensitivity appears to be a general, quantitative attenuation *in vivo* that was observed for all tissues rather than being specific to a cell/organ system.

The diminished growth of hMCMV-ES in different tissues during acute infection severely impairs the study of the HCMV enhancer in this system. To overcome this limitation, we initially attempted to infect adult BALB/c mice with higher doses of hMCMV-ES. Although this level of inoculation permitted more-vigorous replication in the different organs tested (data not shown), it generated technical problems associated with the production of highly concentrated viral stocks. In a search of a more sensitive model of infection, we turned to neonatal

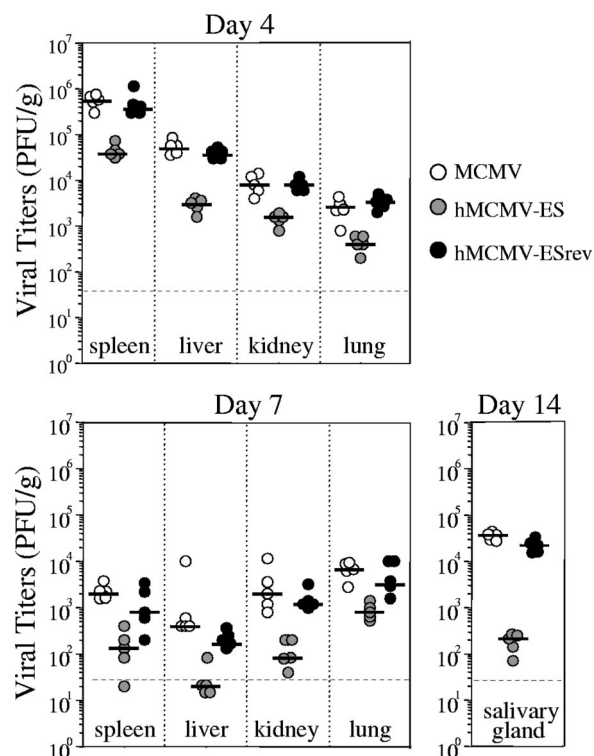


FIG. 2. Growth of hMCMV-ES, parental, and revertant viruses in adult BALB/c mice. Three-week-old BALB/c.ByJ female mice were inoculated i.p. with 1×10^6 PFU per mouse of tissue culture-derived MCMV, hMCMV-ES, or hMCMV-ESrev. Animals were sacrificed at the different times p.i. indicated, selected organs were harvested and sonicated as a 10% (wt/vol) tissue homogenate, and titers were determined by standard plaque assays, including centrifugal enhancement of infectivity (16) on MEF. The dashed line indicates the limit of detection (3×10^1 PFU/g). Horizontal bars indicate the median values. Viral titers in the different organs and times shown were significantly different (P value < 0.05) between groups of animals infected with hMCMV-ES and the parental virus as determined by the Mann-Whitney test (two-tailed).

mice. Work by Reddehase and colleagues (32) has shown that intraperitoneal infection of neonatal BALB/c mice with low doses of MCMV (1×10^2 PFU) leads to significant virus production in different organs and subsequent establishment of latency. Accordingly, we first analyzed the degree of virulence of hMCMV-ES in comparison to that of the parental virus in this system. For this purpose, we challenged groups of 3-day-old BALB/c mice with various doses of hMCMV-ES (ranging from 1×10^3 to 2×10^6 PFU/mouse) or parental and revertant viruses (ranging from 1×10^2 to 1×10^4 PFU/mouse) and evaluated their survival daily for at least a 40-day period. As shown in Fig. 3A, while the estimated 50% lethal dose (LD_{50}) for control viruses was 1.3×10^3 (parental) and 1.4×10^3 (revertant) PFU, the LD_{50} for hMCMV-ES was 2 orders of magnitude higher, 1.7×10^5 PFU, reflecting the significant decrease in virulence associated with hMCMV-ES.

Based on the LD_{50} values determined, and in order to establish conditions that allowed a robust hMCMV-ES infection comparable to that of wild-type MCMV, we proceeded to inoculate 3-day-old BALB/c mice with a viral dose corresponding to 0.3 LD_{50} (5×10^2 PFU/mouse for control viruses and

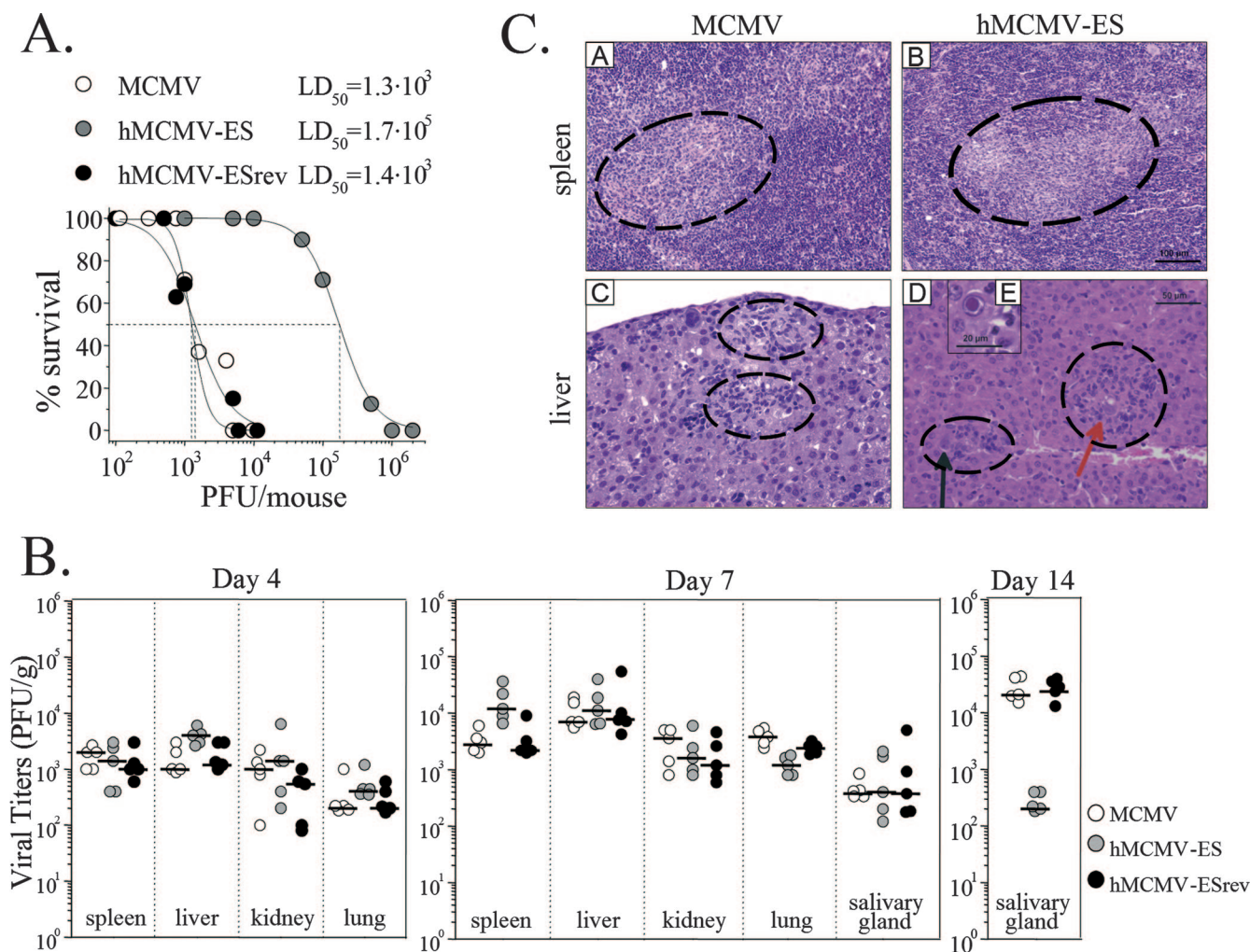


FIG. 3. Infection of neonate BALB/c mice with hMCMV-ES. (A) Virulence level of hMCMV-ES in newborn mice. Groups of 7 to 34 3-day-old BALB/c.ByJ female mice were i.p. inoculated with increasing doses of tissue culture-derived hMCMV-ES (ranging from 1×10^3 to 2×10^6 PFU per mouse) or parental or revertant viruses (ranging from 1×10^2 to 1×10^4 PFU per mouse). Animals were monitored daily for survival until at least 40 days postinoculation. Data points were fitted to a sigmoid function available in Origin software, and the LD_{50} values were estimated from these curves. (B) Growth kinetics of hMCMV-ES in newborn mice. Three-day-old BALB/c.ByJ female mice were i.p. inoculated with tissue culture-derived hMCMV-ES (5×10^4 PFU per mouse), MCMV, or hMCMV-ESrev (5×10^2 PFU per mouse). Mice were sacrificed at different times after infection and specific organs removed. Collected organs were sonicated as a 10% (wt/vol) tissue homogenate, and titers were determined by standard plaque assays, including centrifugal enhancement of infectivity on MEF. All values were among the limits of detection of the assay (5×10^1 PFU). Horizontal bars indicate the median values. The observed differences did not reach statistical difference (P value > 0.05) as determined by the Mann-Whitney test (two-tailed), except for the values for salivary glands at day 14 p.i., with which a significant difference was obtained between the group of animals infected with hMCMV-ES and the group infected with MCMV or hMCMV-ESrev. (C) Histological analysis of livers and spleens from MCMV- and hMCMV-ES-infected newborn mice. Portions of the spleens and livers removed at day 7 p.i. from animals discussed for panel B above were fixed in 4% formaldehyde, processed, and embedded in paraffin by standard procedures. Sections (3 to 4 μ m) were cut with a rotary microtome and stained with hematoxylin and eosin for examination. Shown are sections from spleens of MCMV-infected animals (subpanel A) and hMCMV-ES-infected animals (subpanel B), with regions exhibiting mild lymphoid depletion in the white pulp enclosed by dashed lines. These lesions were frequently associated with discrete inflammatory aggregates mostly constituted by macrophagic cells and occasionally accompanied by eosinophilic intranuclear inclusion bodies. (Subpanels C to E) Liver sections of MCMV-infected mice (subpanel C) and hMCMV-ES-infected mice (subpanels D and E), where moderate inflammatory lesions dispersed through the parenchymata can be recognized (enclosed by dashed lines). Scattered necrotic hepatocytes, cytomegalic cells (red arrow in subpanel D), and Cowdry type A intranuclear inclusion bodies (inset subpanel E and black arrow in subpanel D) could be distinguished in these inflammatory foci. The bars represent 100 μ m in panels A to B, 50 μ m in panels C to D, and 20 μ m in panel E.

5×10^4 PFU/mouse for hMCMV-ES). We then evaluated the growth of hMCMV-ES in comparison to that of the control viruses in the major target organs for CMV at different days p.i. Under these conditions, hMCMV-ES grew to levels equivalent to and followed a course similar to those of the parental

and revertant viruses in spleen, liver, lungs, kidneys, and salivary glands at days 4 and 7 after infection (Fig. 3B). Reduced viral yields (compared to those from mice infected with the corresponding controls) were found only in the salivary glands of hMCMV-ES-infected animals 14 days after inoculation. Di-

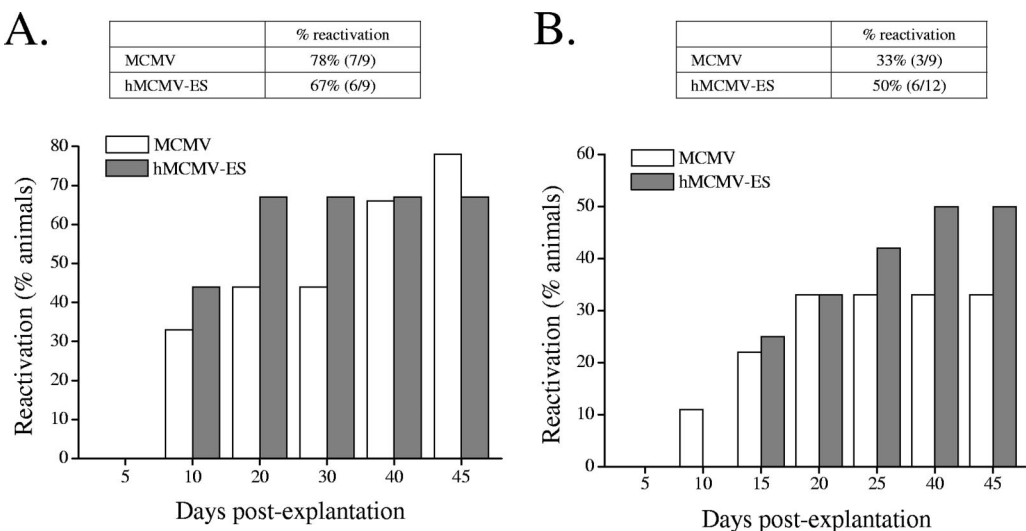


FIG. 4. Reactivation of hMCMV-ES virus. Groups of 9 to 12 3-day-old BALB/c.ByJ female mice were i.p. inoculated with tissue culture-propagated hMCMV-ES (5×10^4 PFU per mouse) or the parental virus (5×10^2 PFU per mouse) and maintained for 4 months to establish latency. Animals were sacrificed and their spleens, lungs, and salivary glands harvested. Reactivation was examined from spleens and lungs, which were manually minced and placed with medium in wells of a six-well plate. Cultures were kept (with fresh medium addition when required) for 50 days. Every 5 days, part of the supernatant was transferred to MEF monolayers (a centrifugal enhancement of infectivity step was included) for detection of infectious virus. Graphics show the cumulative frequencies of reactivation events over time from spleen (A) and lung (B) explants. The percentages of reactivation accumulated at day 50 postexplantation are indicated in the corresponding tables, with the number of positive mice versus the number of total animals per group shown in parentheses. The identities of the reactivated viruses (which differ within groups in the sizes of the HCMV and MCMV enhancer regions) were confirmed by PCR analysis using two primers that bind to viral sequences flanking the MCMV enhancer and as template viral DNAs from the indicator MEF cultures when a cytopathic effect was reached (data not shown). In addition, PCR products from a representative sample of each group and organ were cloned and sequenced. Salivary glands were analyzed to ensure the clearance of infectious virus at the time of explantation by sonicating them as a 10% (wt/vol) tissue homogenate and subsequently transferring them to MEF monolayers. Data shown correspond to results from one of two independently performed experiments in which comparable results were obtained. Percentages of reactivation between MCMV and hMCMV-ES were not statistically different, as determined by the chi-square test (two-tailed).

rect inoculation of the chimeric virus in the submaxillar gland did not ameliorate this partially impaired growth (data not shown), thus suggesting a particular defect of hMCMV-ES in replicating in this gland more than an incapacity of the mutant virus to successfully reach it. Therefore, except in the salivary glands, conditions were established in the neonatal murine model that allowed hMCMV-ES to disseminate and replicate in relevant organs involved in acute infection. In agreement with the viral titers obtained, signs of MCMV-associated pathology were observed in the different organs analyzed from hMCMV-ES-infected animals and were more prominent in spleens and livers than in other organs. Figure 3C shows some of the lesions observed in these two organs. While, quantitatively, lesions in the spleens of hMCMV-ES- and MCMV-infected mice were very similar, slightly more frequent and florid lesions were observed in the livers of animals inoculated with the virus with the replaced enhancer.

We next examined whether, under these experimental conditions, hMCMV-ES was capable of establishing latency and subsequently reactivating from this state. Groups of 9 to 12 3-day-old mice were infected with 5×10^4 PFU of hMCMV-ES or 5×10^2 PFU of the parental virus. After 4 months, when persistent virus could no longer be detected in salivary gland homogenates, viral reactivation was investigated with splenic and lung explants as described by Jordan and Mar (21) and Presti et al. (31), systems that have proven to be more efficient and reproducible than in vivo reactivation techniques.

As shown in Fig. 4, reactivation in spleens was detected in 78% of animals infected with the parental virus and in 67% of animals infected with hMCMV-ES. The frequency of reactivation of hMCMV-ES in the lungs, although lower than in spleens, was also similar to that exhibited by the parental virus (50% and 33% for the mutant and the parental virus, respectively). In addition, in both organs, the reactivation of the two viruses followed roughly similar kinetics. Thus, under the experimental conditions established, the efficiency and dynamics of reactivation of hMCMV-ES in explant cultures were comparable to those of the parental virus.

The exchange between enhancers of different CMV species results in hybrid viruses with similar or slightly diminished in vitro replication abilities but significantly altered dissemination capacities and/or host cell tropisms (2, 14, 18, 34). These observations highlight the biological significance of this regulatory region, most likely adapted during coevolution to suit species-specific niches. In order to develop a system that allows the study of the HCMV enhancer in the different settings in vivo, we have used here the hMCMV-ES virus and the neonate mouse model and established conditions of infection that mostly reproduce the extent of viral replication in different organs and parallel latency/reactivation properties of parental MCMV. Until now, the study of particular genetic elements of the HCMV enhancer in the context of the complete viral genome has been restricted to a few tissue culture systems (5, 9, 15, 19, 22, 29). Thus, for the first time, it is possible to

ascertain the roles of selective HCMV enhancer binding elements, cellular factors, and signaling pathways operating through this region in an intact physiological system, in the establishment and maintenance of latency, and in the control of the reactivation events in the context of an *in vivo* infection. Knowledge of the mechanisms governing enhancer function may lead to new strategies for controlling primary and recurrent HCMV infections, with the small-animal model system established here being a valuable tool for their evaluation.

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